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Crohn's Disease: Loss of Tolerance or a Disorder of Autophagy?

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Key Words
Autophagy · Bacteria · Crohn's disease · IL-10 · Intestinal macrophages · TLR4

Abstract
Crohn's disease (CD) is characterized by a breakdown of the intestinal epithelial barrier function leading to an uncontrolled immune response to bacterial antigens. Available data demonstrate that appropriate response and early host defense against invading bacteria are crucial to maintain tolerance towards commensal bacteria. When the mechanisms of early removal of invading bacteria are disturbed, a loss of tolerance and a full-blown adaptive immune reaction, which is mounted against the usually harmless commensal flora, are induced. Dysfunction of autophagy caused by genetic variations within CD susceptibility genes, such as ATG16L1 and IRGM, results in defective handling of intracellular and invading bacteria and causes prolonged survival and defective clearance of those microbes. Dysfunction of ATG16L1 and IRGM has also been shown to cause aberrant Paneth cell function and uncontrolled secretion of proinflammatory cytokines finally resulting in increased susceptibility to bacterial infection and the onset of colitis. Interestingly, autophagy can also be regulated by other CD susceptibility genes, such as NOD2 (nucleotide oligomerization domain 2) or PTPN2 (protein tyrosine phosphatase nonreceptor type 2) and the presence of the CD-associated variations within these genes results in similar effects. Taken together, more and more evidence suggests a close functional correlation between loss of tolerance and defective autophagy in CD patients. Therefore, most likely, the onset of CD is triggered by both a loss of tolerance as well as a dysfunction of autophagy, which finally results in the onset of chronic intestinal inflammation.
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Introduction

Along with ulcerative colitis (UC), Crohn's disease (CD) is one of the major forms of inflammatory bowel disease (IBD). The interplay between environmental factors, genetic predisposition, intestinal microbiota as well as the innate and adaptive immune system needs to be tightly controlled. An imbalance in these factors predisposes to the pathogenesis of IBD. Current hypotheses suggest that an epithelial barrier defect coupled with an impaired immune response of the innate as well as adaptive immune system to the commensal flora results in either excessive upregulation or impaired suppression of inflammatory responses, finally leading to chronic intes-

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tinal inflammation [1]. Current studies suggested that intestinal microbial diversity is reduced and species composition is altered, especially in CD: Firmicutes are reduced while Bacteroidetes and Enterobacteriaceae are increased in the intestine of CD patients [2, 3]. Genome-wide association studies identified genetic variations in 163 genes which are associated with IBD, including 110 loci shared by both disease subtypes, 30 loci classified as specific for CD and 23 as specific for UC [4]. Of note, members of the innate immune system, such as the intracellular bacterial-sensor NOD2 (nucleotide oligomerization domain 2) as well as the autophagy genes ATG16L1 (autophagy-related gene 16-like 1) and IRGM (immunity-related GTPase family M), are exclusively associated with CD [4].

CD – Loss of Tolerance?

Immune cells trigger an effective inflammatory response as soon as pathogen-associated molecular patterns (PAMPs) are present. In the gut, however, myriads of commensal bacteria or food antigens do normally not provoke adverse reactions. This seems to be due to active tolerance induction by intestinal epithelial cells (IECs) and specific immune cell subsets. IECs secrete mucins and antimicrobial molecules to limit the number of commensals directly penetrating the gut mucosa [5]. In CD, this barrier is impaired and increased translocation of bacteria and food antigens provokes an enhanced proinflammatory response of the innate as well as adaptive immune system, which finally results in loss of tolerance towards commensal antigens [6, 7].

Intestinal Macrophages

Myeloid intestinal immune cells, such as intestinal macrophages (iMACs) and dendritic cells (DCs) – in contrast to macrophages/DCs in other tissues, display a hypo-responsive, tolerance-inducing phenotype [8]. iMACs from CD patients, however, display a significantly higher expression of CD14, CD16, HLA-DR, CD11b, and CD11c, indicating additional macrophage populations in the inflamed mucosa, which reflects either a recruitment of new cells from the blood or a phenotypic change in resident cells [9, 10]. Under normal conditions, iMACs can eliminate intracellular bacteria without provoking an immune reaction [11]. They sample the luminal content and, by expression of specific surface markers and cytokine secretion, induce tolerogenic T cells [8]. These iMACs from normal mucosa rarely express the costimulatory molecules CD80 and CD86. In CD, a macrophage population is found with high expression of costimulatory molecules, which is presumably responsible for the perpetuated immune response [12]. This special population of CD iMACs also reveals increased activation of nuclear factor (NF)-κB in the inflamed mucosa [13], which might, at least in part, be due to decreased levels of human glucocorticoid receptor in mucosal CD iMACs and lead to decreased protection against NF-κB action [14]. Further, NADPH oxidase mRNA is downregulated or absent in macrophages from normal mucosa, which correlates with a lack of oxidative burst activity. In CD, however, macrophage oxidative burst activity is increased and NADPH oxidase mRNA is induced [15]. CD iMACs also display increased expression of TLR2 and TLR4, which likely contributes to their increased activation status in response to PAMPs. The absence of TLRs abolishes the reactivity of mucosal macrophages to bacterial wall products, while the presence of TLRs might contribute to the inflammatory process [16]. Of note, the chaperone molecule glycoprotein gp96 links the adaptive with the innate immune system. During cellular stress, peptide-loaded gp96 can be released and presented to T cells by antigen-presenting cells. gp96 is induced during differentiation of normal iMACs but is not detected in iMACs in CD mucosa. As gp96 has been described as having a role in tolerance induction, this may be relevant for loss of tolerance against luminal bacteria found in CD patients [17]. Further, a reduced expression of subunits of the ubiquitin-proteasome system in iMACs of normal mucosa supports the concept of the presence of a nonreactive, anergic macrophage phenotype in the gut under normal conditions. Reinduction in iMACs of IBD mucosa reflects activated iMACs which can present antigenic peptides and thus support inflammation [18].

Immune Cells and Pattern Recognition Receptors

As it is the case for IECs, NOD2 malfunction in DCs and iMACs results in poor bacterial clearance [6, 19] and consequently in prolonged bacterial infection and hyperactivation of the immune system [20], finally promoting the secretion of proinflammatory cytokines and an immune-activating phenotype. NLRP3 and NLRP6 are able to form multiprotein complexes called inflammasomes, which are important for processing pro-IL-1β and pro-IL-18 into their mature forms and their final secretion [21]. Low-level inflammasome activation and IL-18 secretion is essential for wound healing and repair in the

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intestines [21]. Physiologic NLRP3 and NLRP6 activity is involved in shaping the composition of the intestinal microbiota [22, 23]. However, uncontrolled inflammasome activity results in enhanced proinflammatory responses and proliferation of destructive immune cells [21].

Several proteins are involved in the modulation of intracellular signaling pathways downstream of pattern recognition receptors and the outcome of the resulting immune response. The CD risk gene PTPN2 (protein tyrosine phosphatase nonreceptor type 2) [24] negatively controls the activation of proinflammatory signaling cascades [25, 26]. If PTPN2 is lost or – as in some CD patients – malfunctioning, it results in hyperactivation of monocytes by bacterial products [27] and increased responses to the proinflammatory cytokines TNF and IFN-γ [26, 28]. Finally, an inflammatory response with increased levels of IL-6, IL-12, and IFN-γ as well as a loss of intestinal barrier function is the result, leading to the breakdown of tolerance mechanisms [26]. PTPN22 also controls bacterial-induced signaling cascades [29] and a variant within the PTPN22 gene locus protects from CD [30]. In CD, intestinal levels of PTPN22 are reduced, resulting in enhanced secretion of IL-6 but reduced IL-12 secretion [31]. This leads to a skewed response to pathogens inducing a cytokine profile favoring pathogenic Th17 cell proliferation.

At a functional level, alterations in pattern recognition receptor signaling in innate immune cells result in the aberrant induction of adaptive immune responses. During intestinal inflammation, intestinal CD14+ cells secrete enhanced levels of IL-1β, IL-6, and IL-12 and thus promote the development of Th1 and Th17 cells [32]. Th17 cells, which defend extracellular bacteria and fungi in healthy persons [33], are increased in IBD patients and play a major role in driving pathologic inflammation and tissue destruction in the intestine [34]. In the intestinal T cell population, high proportions of so-called inducible regulatory T cells (T_{reg}) can be found [35]. They actively downmodulate excessive activation of the adaptive immune system. In contrast to natural T_{reg}, inducible T_{reg}s develop in the periphery upon antigen challenge and relieve inflammatory reactions after antigen clearance following an infection [36]. In the gut, microbial-specific inducible T_{reg}s are believed to induce tolerance towards commensal microflora [37]. In CD, however, this tolerance-inducing mechanism seems to be lost: The tight junctions between epithelial cells become leaky [7], and consequently increased numbers of commensal bacteria can penetrate the mucosa finally and thus increase NOD2 activation [6]. Further, high numbers of activated proinflammatory T cells can be found [38]. Additionally, IL-10 is an important regulatory cytokine able to suppress the synthesis of proinflammatory mediators, including IFN-γ and TNF, as well as costimulatory molecules and MHC class II molecules on antigen-presenting cells [39]. T_{reg}s and to a lower extent also Th2 cells produce IL-10 in order to downregulate proinflammatory responses and end an immune reaction restoring tolerance towards commensal bacteria [39]. The importance of this tolerance-inducing functions of IL-10 is highlighted by the fact that loss of IL-10 results in spontaneous colitis in mice [40], and loss-of-function variants in either IL-10 or IL-10 receptors result in severe, very-early-onset IBD [41].

CD – Dysfunction of Autophagy?

Autophagy is critically involved in regulating innate immune responses by providing a defense mechanism against intracellular microbial pathogens and mediating antigen presentation via MHC class II molecules [42, 43]. Dysfunctional autophagy is associated with defective bacterial handling, prolonged intracellular survival of pathogens, and uncontrolled inflammation. Levels of autophagy proteins, such as ATG16L1 or IRGM, are significantly decreased in the intestines of CD patients [44]. To date, three autophagy genes have been confirmed as CD susceptibility genes, namely ATG16L1, IRGM, and LRRK2 (leucine-rich repeat kinase 2) [24, 45]. LRRK2-deficient mice are more susceptible to dextran sodium sulfate (DSS)-induced colitis, since LRRK2 inhibits activation of NFAT1, which promotes the secretion of proinflammatory cytokines [46]. LRRK2 expression is elevated in intestinal tissue of CD patients, and LRRK2-deficient cells are impaired in killing of intracellular bacteria [47], which suggests a possible role for LRRK2 in CD pathogenesis.

ATG16L1, Interacting Molecules, and Paneth Cell Function

ATG16L1 represents a key molecule within the autophagy network which is responsible for subcellular localization of the autophagy machinery [48]. Presence of the SNP rs2241880 within the gene locus encoding ATG16L1 results in substitution of threonine by alanine (T300A) and has been strongly associated with an increased risk for developing CD [24]. Recent studies demonstrated that the presence of the CD-associated ATG16L1 variant results in increased numbers, pro-

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longed survival, and elevated replication of intracellular bacteria [42, 43] and consequently elevated secretion of the proinflammatory cytokines TNF and IL-6, which promote inflammatory conditions in the intestine [49]. Presence of the ATG16L1 variation obviously only affects the extent of autophagy following activation, while basal levels of autophagy remain unaffected. A recent study demonstrated that CD patients featuring the ATG16L1 variation exhibit impaired antigen uptake and processing as well as a defective interaction between DCs and IECs in the intestine [50]. In a study using mice lacking the conserved coiled-coil domain of ATG16L1, increased inflammasome activation in response to PAMPs causing enhanced secretion of IL-1β and IL-18 was found and the mice were more susceptible to DSS-induced colitis [51]. Recent studies identified a role for microRNAs in downregulating ATG16L1 and autophagy resulting in prolonged bacterial persistence [52, 53].

ATG16L1 seems to exert a close functional correlation with other IBD susceptibility genes, such as NOD2 or PTPN2, in regulating autophagy. In DCs, NOD2-mediated autophagy is crucial for handling of invading bacteria as well as for antigen presentation and the induction of antigen-specific CD4+ T-cell responses via MHC class II molecules. DCs from CD patients carrying either the CD-associated ATG16L1 or NOD2 variations are defective in autophagosome formation, bacterial trafficking, and antigen presentation [42]. As a possible mechanism, NOD2 is crucial for the initiation of autophagy by recruiting ATG16L1 to the cell membrane at the site of bacterial entry. Cells featuring CD-associated NOD2 polymorphisms are unable to direct ATG16L1 to the plasma membrane and are deficient in handling of invading bacteria [43]. Presence of the CD-associated ATG16L1 variant resulted in increased secretion of the proinflammatory cytokines IL-1β and IL-6 from peripheral blood mononuclear cells of CD patients in response to stimulation with NOD2 ligands [54]. These observations strongly suggest that NOD2 as well as autophagy play a key role for the innate immune system and present the functional mechanism how dysfunction of autophagy could essentially contribute to the onset of chronic intestinal inflammation. A further close functional correlation exists between PTPN2, ATG16L1, NOD2, and autophagy in general. PTPN2 expression is, at least partially, regulated by ATG16L1 and NOD2. At a functional level, PTPN2 is involved in NOD2-mediated autophagy, since presence of the CD-associated PTPN2 variations results in impaired autophagosome formation and is associated with decreased expression of a number of autophagy genes, including IRGM and ATG16L1 [27, 44]. Of note, a further protein tyrosine phosphatase, namely PTPN22, has also been demonstrated to regulate NOD2-mediated autophagy [29].

Besides its role in the handling of invading pathogens, ATG16L1 has been critically associated with the function of Paneth cells located within the crypts of Lieberkühn in the small intestine. Paneth cells represent a specialized epithelial cell type, which is important for the secretion of antimicrobial factors into the intestinal lumen via the production and secretion of characteristic cytoplasmic granules. In particular, hypomorphic ATG16L1 ($ATG16L1^{HM}$) mice lack the antibacterial enzyme lysozyme in the mucus of ileal sections, and lysozyme was diffusely detectable in a number of Paneth cells in $ATG16L1^{HM}$ mice accompanied by aberrant, disorganized granules as well as decreased numbers of granules [55]. Paneth cells from $ATG16L1^{HM}$ mice further revealed increased expression of proinflammatory molecules, such as the adipocytokines leptin and adiponectin [55]. Of special interest, Paneth cells derived from CD patients homozygous for the ATG16L1 CD risk allele feature similar abnormalities in Paneth cell morphology and granule secretion as $ATG16L1^{HM}$ mice and endoplasmic reticulum stress even during quiescent CD-associated bacterial persistence in the intestine [55, 56]. Interestingly, all of the mentioned abnormalities in Paneth cell morphology and function were absent when $ATG16L1^{HM}$ mice were raised in an enhanced barrier facility, but could be completely introduced again following infection of the $ATG16L1^{HM}$ mice with the murine norovirus strain CR6 for 7 days, which indicates a critical role for a virus-plus-susceptibility gene interaction [57]. $ATG16L1^{HM}$ mice only displayed a severe intestinal injury response to DSS treatment when they were infected with CR6 at least 7 days before DSS administration, but not in the absence of the virus or when the virus infection occurred at the same time as the DSS treatment initiation. These observations suggest that virus-plus-susceptibility gene interactions in addition to environmental factors and commensal bacteria are critically involved in the pathogenesis of chronic intestinal inflammation.

IRGM

Several variations within the IRGM gene locus are associated with CD, e.g. the coding region of IRGM, a 20-kb deletion polymorphism upstream of the IRGM transcriptional start site, affects several transcription factor binding sites and hereby impairs IRGM expression levels and

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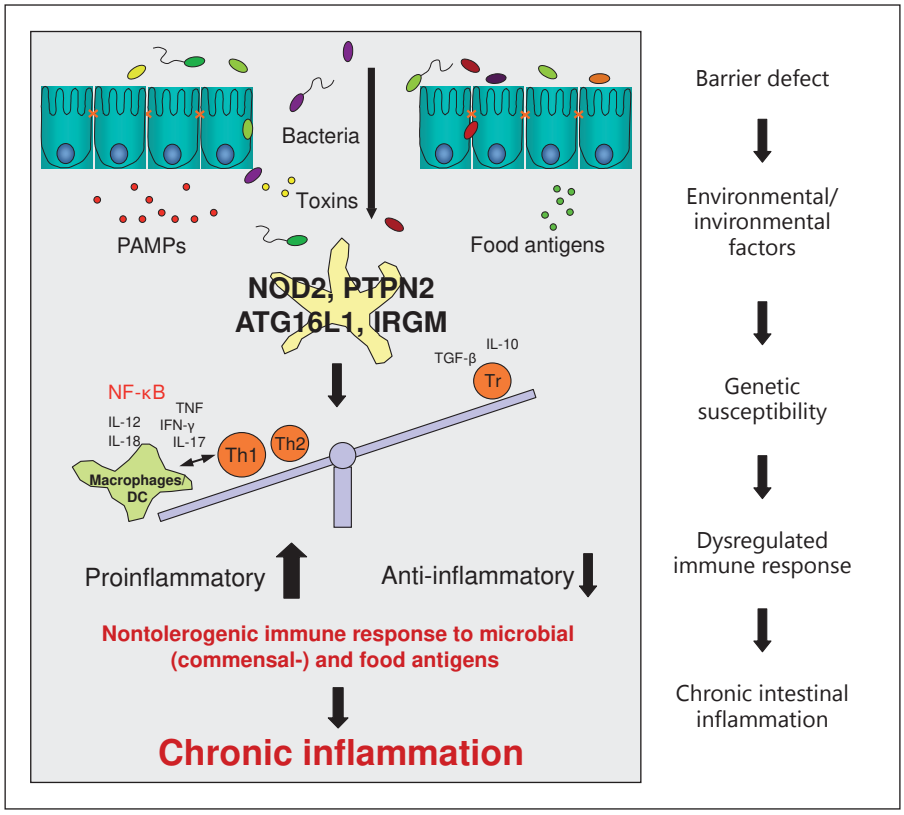

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Fig. 1. CD pathogenesis: an epithelial barrier defect favors the invasion of commensal and pathogenic bacteria, food antigens, and toxins into the gut mucosa. The presence of genetic variations, such as in autophagy-related genes or pattern recognition receptors, contributes to the barrier defect and causes an impaired immune response. Hereby, alterations in tolerance mechanisms affecting the innate and adaptive immune system as well as in autophagy can be observed. Of particular interest hereby seems to be CD-specific alterations in intestinal macrophages. The aberrant immune response results in an imbalance between pro- and anti-inflammatory cytokines finally establishing the onset of chronic intestinal inflammation.

the promoter region or the 5′-untranslated region of IRGM [24, 58, 59]. Reduced expression of IRGM seems to be responsible for the decreased function of IRGM and a subsequent impairment in autophagy, which suggests that a certain level of IRGM protein is necessary for proper protein function. The necessary threshold level can obviously not be reached in the presence of the CD-associated polymorphisms and lower levels of IRGM expression have been detected in lymphocytes from CD patients [58]. Interestingly, a recent study demonstrated that the microRNA family, miR-196, which is overexpressed in the inflamed intestinal epithelium of CD patients, causes downregulation of the protective IRGM variant, but does not affect levels of the disease-associated variant. The resulting decrease in IRGM expression levels contributes to impaired autophagy and enhanced intracellular bacterial replication [60]. It has been shown that functional IRGM1 plays a key role in regulating the maturation of pathogen-containing vacuoles, as well as the adhesion and motility of activated macrophages. Consequently, IRGM1 deficiency results in increased susceptibility of mice to pathogens and finally systemic infection [61–63]. IRGM is also crucial for IFN-γ-mediated autophagy and the elimination of intracellular *Mycobacterium tuberculosis* from human macrophages [64]. siRNA-induced knockdown of IRGM in human cells results in a defect in the autophagy machinery favoring the persistence of bacteria resulting in increased proinflammatory responses [49, 65]. Interestingly, IRGM-deficient mice featured aberrant Paneth cell morphology and localization, which is associated with impaired autophagy and increased susceptibility to DSS-induced colitis [66].

Conclusion

Taken together, available data demonstrate that appropriate response and early host defense against invading bacteria is crucial to maintain tolerance towards commensal bacteria. If the mechanisms of early removal of invading bacteria are disturbed, this leads to a loss of tolerance and a full-blown adaptive immune reaction, which is mounted against the usually harmless commensal flora. Additionally, a number of studies clearly provide evidence for the involvement of dysfunctional autophagy in the pathogenesis of CD. Dysfunction of autophagy caused

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by genetic variations within CD susceptibility genes results in defective handling of intracellular and invading bacteria as well as aberrant Paneth cell function and uncontrolled secretion of proinflammatory cytokines and thus in increased susceptibility to bacterial infection and the onset of colitis. However, more and more evidence suggests a close functional correlation between loss-of-tolerance and defective autophagy in CD patients, and all of these effects can be observed during human CD (fig. 1). Therefore, most likely, the onset of CD is dependent on both a loss of tolerance as well as a dysfunction of autophagy, which finally results in the onset of chronic intestinal inflammation in humans.

Disclosure Statement

The authors declare that no conflicts of interest exist.

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